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### Designing and Testing of a Health-Economic Markov Model for Prevention and Treatment of Early Psychosis

Wijnen, Ben F. M.; Thielen, Frederick W.; Konings, Steef; Feenstra, Talitha; Van der Gaag, Mark; Veling, Wim; De Haan, Lieuwe; Ising, Helga; Hiligsmann, Mickael; Evers, Silvia M. A. A.

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# Smoothened-dependent and -independent pathways in mammalian noncanonical Hedgehog signaling

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Edited by Xiao-Fan Wang

Hedgehog proteins are pivotal morphogens acting through a canonical pathway involving first activation of ligand binding to Patched followed by alleviation of Smoothened receptor inhibition, leading to activation of Gli transcription factors. Noncanonical Hedgehog signaling remains poorly characterized but is thought to be mainly dependent on Smoothened. However, Smoothened inhibitors have yielded only partial success in combating Hedgehog signal transduction-dependent cancer, suggesting that noncanonical Smoothened-independent pathways also are clinically relevant. Moreover, several Smoothened-dependent effects (e.g. neurite projection) do not require transcriptional activation, further suggesting biological importance of noncanonical Smoothened-dependent pathways. We comprehensively characterized the cellular kinome in Hedgehog-challenged murine WT and Smoothened<sup>-/-</sup> fibroblasts as well as Smoothened agonist-stimulated cells. A peptide assay-based kinome analysis (in which cell lysates are used to phosphorylate specific kinase substrates), along with endocytosis, Lucifer Yellow-based, and immunoblotting assays, identified an elaborate signaling network of both Smoothened-dependent and -independent pathways that mediates actin reorganization through Src-like kinases, activates various proinflammatory signaling cascades, and concomitantly stimulates Wnt and Notch signaling while suppressing bone morphogenetic protein (BMP) signaling. The contribution of noncanonical Smoothened-independent signaling to the overall effects of Hedgehog on cellular physiology appears to be much larger than previously envisioned and may explain the transcriptionally independent effects of Hedgehog signaling on cytoskeleton. The observation

that Patched-dependent, Smoothened-independent, noncanonical Hedgehog signaling increases Wnt/Notch signaling provides a possible explanation for the failure of Smoothened antagonists in combating Hedgehog-dependent but Smoothened inhibitor-resistant cancer. Our findings suggest that inhibiting Hedgehog–Patched interaction could result in more effective therapies as compared with conventional Smoothened-directed therapies.

Cell fate is determined by morphogens, molecules whose nonuniform distribution governs the pattern of tissue development (1, 2). Notable examples of morphogens include Hedgehog, Wingless-related integration site (Wnt),<sup>5</sup> and bone morphogenetic protein (BMP) (3–5). The intracellular signaling resulting from engagement of morphogens with their cognate receptors is involved in many physiological and pathophysiological processes, including embryogenesis, tissue regeneration, and carcinogenesis. Fully understanding morphogen signaling is therefore of the utmost importance (6). Unfortunately, morphogen signaling is often extremely complex, a special case in point being signal transduction initiated by Hedgehogs (7).

Hedgehog proteins are a highly conserved family of intercellular signaling molecules. Originally identified as a *Drosophila* segment polarity gene required for embryonic patterning, several vertebrate homologues have been discovered—Indian (Ihh), Desert (Dhh), and Sonic Hedgehog (Shh), the latter being most extensively characterized (8). Hedgehog signals are fundamental regulators of embryonic development, as illustrated by embryological malformations seen when accurate timing of Hedgehog signals during gestation is corrupted (9). Hedgehog remains active in the post-embryonic period, maintaining histostasis in a variety of tissues, including the gastrointestinal

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This article contains Table S1.

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<sup>5</sup> The abbreviations used are: Wnt, Wingless-related integration site; BMP, bone morphogenetic protein; Dhh, Desert Hedgehog; Fu, fused kinase; Gli, glioma-associated oncogene; Ihh, Indian Hedgehog; Shh, Sonic Hedgehog; SuFu, suppressor of fused protein; ROCK, Rho-associated coiled-coil-containing protein kinase; MEF, mouse embryonic fibroblast; mTOR, mammalian target of rapamycin kinase; PKA, protein kinase A; PKC, protein kinase C; Pak, p21-activated kinase; PKB, protein kinase B; SAG, smoothened agonist; PVDF, polyvinylidene difluoride; Ptc, Patched.

tract and the immune system (10). Continuous hedgehog signaling is an essential permissive factor for many cancers and causative in basal cell carcinoma of the skin (11). In humans, one-allelic loss of the inhibitory hedgehog receptor Patched is sufficient to produce the so-called Gorlin syndrome (12), which is associated with rhabdomyosarcoma and the development of multiple basal cell carcinomas.

Despite the importance of Hedgehog signaling for human physiology and pathophysiology, the molecular details underlying this signaling pathway remain only partly characterized. The primary receptor for Hedgehogs is Patched, an unconventional receptor, as it does not convey the Hedgehog signal to the intracellular components of the pathway itself. Rather, binding of Hedgehog to Patched alleviates the inhibitory effect of Patched on another membrane receptor, Smoothened. The Patched inhibition alleviation is probably caused by internalization of Patched following Hedgehog binding, but the signaling mechanisms involved remain obscure (13). Subsequently, Smoothened mediates the activation of the latent transcription factor glioma-associated oncogene (Gli) via a process which involves the kinase Fused (Fu), the Suppressor of Fused protein (Su(Fu)) (14, 15), and inhibition of Gli proteolysis. Gli proteins are considered the final transcriptional effectors of Hedgehog signaling, both in normal vertebrate development and in oncological disease (16). Together, this signaling cascade may be termed the canonical Hedgehog pathway. It is obvious that enhanced knowledge of the signaling elements involved in this pathway should prove exceeding useful in defining novel rational therapy directed at disease emanating from aberrant activation of canonical Hedgehog signaling.

In addition to canonical Hedgehog signaling, a role for transcription-independent signaling via Hedgehog has also been suggested (17–19). Tantalizingly, the presence of canonical and noncanonical Hedgehog signaling opens the theoretical possibility to uncouple the anti-cancer effect of Hedgehog signaling on cancer in general (20) and the trophic effect of Hedgehog signaling on specifically cancer stem cells. In the absence, however, of knowledge on the molecular pathways that mediate these noncanonical effects of Patched-dependent but Smoothened-independent Hedgehog signaling, this possibility remains hypothetical only. In an effort to address this issue, here we endeavor to characterize the signaling pathways involved.

## Results

### *Hedgehog stimulation provokes rapid and marked reorganization of the cellular kinome*

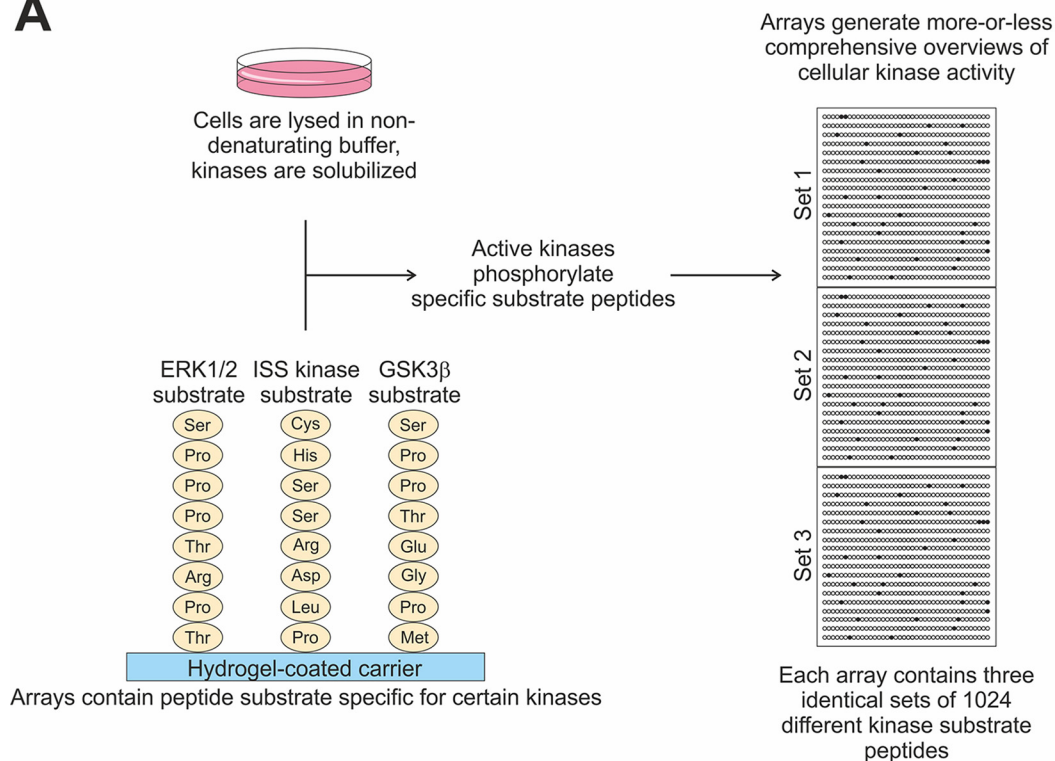
We set out to characterize the kinase activities associated with Hedgehog challenge in general, as well as those specifically associated with Patched activation or Smoothened activation in isolation. To this end, we exploited the power of peptide array-based kinome profiling, which allows the generation of comprehensive descriptions of cellular kinase activities (21–23). The general approach to this study, both technically and biologically, is shown in Fig. 1. We characterized the kinase signatures associated with Hedgehog stimulation of mouse embryonic fibroblasts (MEFs), which we have recently shown to constitute a powerful model for delineating signal transduction

events (24). We established that under our experimental conditions, these cells do not endogenously release Hedgehog (not shown). Cells were incubated for 10 min with either 2  $\mu$ g/ml Shh or a vehicle control, and the cell lysates were employed for *in vitro* phosphorylation of peptide arrays using [ $\gamma$ - $^{33}$ P]ATP. Arrays consisted of 1024 different undecapeptides, of which 48 are various technical controls, whereas the remaining 976 peptides provide kinase substrate consensus sequences spanning the entire mammalian kinome and have been shown by us earlier to provide comprehensive insight in cellular signal transduction (25). On each separate carrier, the array was spotted three times, to allow assessment of possible variability in substrate phosphorylation. As a control for the specificity of the reaction, [ $\gamma$ - $^{33}$ P]ATP was used; no incorporation of radioactivity was seen (data not shown). We then calculated the mean phosphorylation level for all substrates before and after the treatment (total number of data points was 9 for each group). The technical quality of the profiles was good, and we only allowed experiments in which the Pearson product moment correlation coefficient was more as 0.95 for the technical replicates. Results were collapsed on elective signal transduction categories (see “Experimental procedures” and Ref. 25).

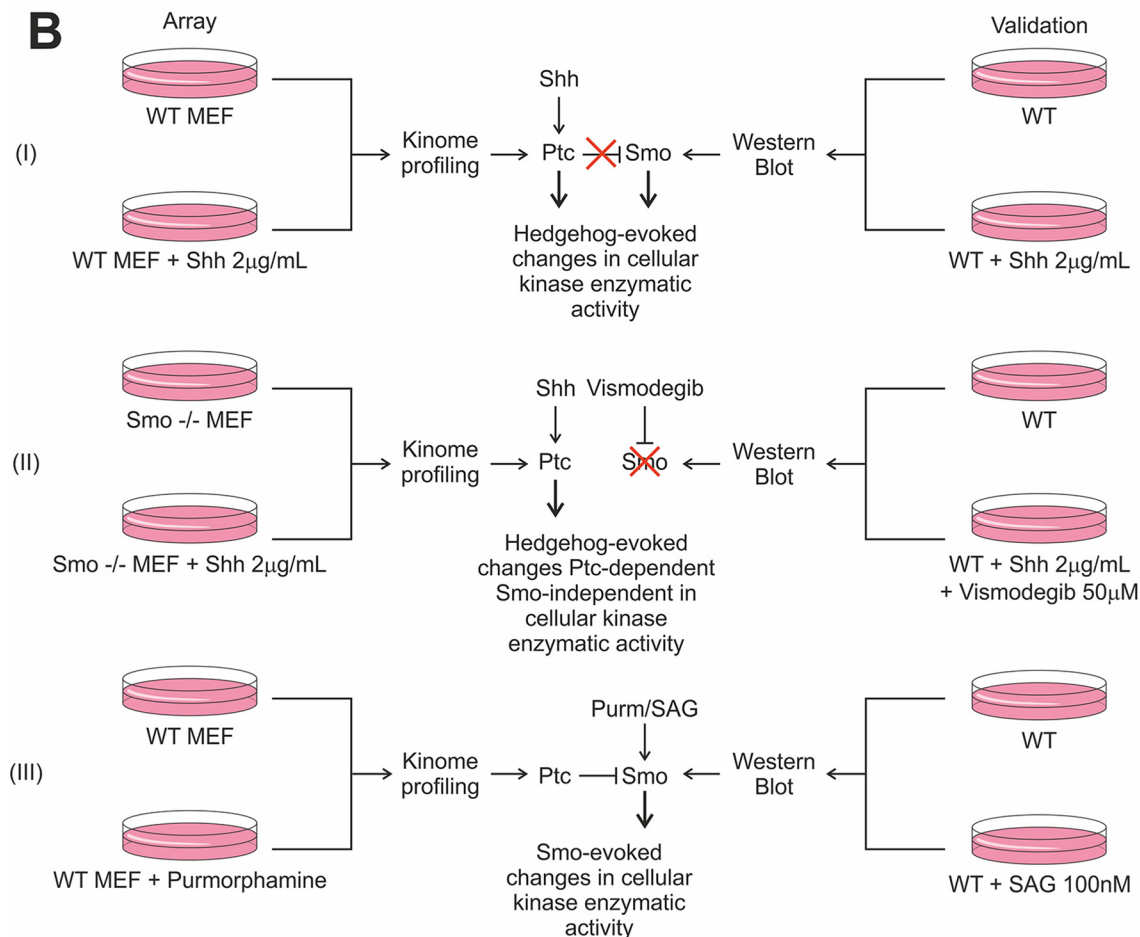
The results are shown in Fig. 2A and detailed in Table S1. They show that Hedgehog challenge provokes fast and substantial remodeling of cellular signaling. Particularly notable is the up-regulation of mTOR signaling. mTOR is a key component of Hedgehog signaling and is a putative target for treating Hedgehog-driven cancers (26). Other interesting points include an up-regulation of G protein-coupled receptor kinase enzymatic activity, which is able to control Smoothened activity (27, 28). This is also in line with the fact that Smoothened itself is such a receptor, and the observation that PKC enzymatic activity is up-regulated confirms the canonical mode of action of G protein-coupled receptors. Strong regulation of PKA, a proposed regulator of Hedgehog signaling (29), is also seen. We observed activation of a variety of pro-inflammatory signaling modules (including Lyn, Fyn, and peptides that are consensus substrates for Bruton's tyrosine kinase), but as embryonic fibroblasts are not immunological cells, the importance of this observation is uncertain. In our untransformed epithelial model system, Hedgehog stimulation reduced Wnt signaling. These data are in line with studies showing that Hedgehog acts as an inhibitor of Wnt signaling in colon cells (30) although an activating role for Hedgehog on Wnt signaling has been proposed in cancer stem cells (31). Last, the up-regulation of substrate peptides for p21-activated kinase (Pak) activity and related molecules indicates that Hedgehog stimulation stimulates actin reorganization and morphological changes. Together, these data show that the effect of Hedgehog on the cellular kinome is rapid and profound.

Despite the great sensitivity and efficiency of array kinome profiling, we validated several of the key pathways by Western blotting (Fig. 2B). Consistent with canonical Shh signaling, phosphorylation of PKC was observed (intensity of  $\alpha$ -phospho-PKC $\delta/\theta$  increased by a factor of 1.22), showing the validity of these models. Second, we show an increased activity of the mTOR-PKB/Akt-S6 pathway upon Shh stimulation (intensity of  $\alpha$ -phospho-Akt staining increased by a factor of 1.75,  $p$  <

**A**



**B**





0.05). Furthermore, in agreement with the Shh-induced cytoskeletal remodeling seen in kinome experiments, we observed an increase in cofilin (intensity of  $\alpha$ -phospho-cofilin staining increased by a factor of 1.86,  $p < 0.05$ ) and Src family phosphorylation (intensity of  $\alpha$ -phospho-Src staining increased by a factor of 1.19). Although these changes in phosphorylation are more modest than those observed in the kinome array, they do support the peptide array data. As Western blotting measures the sum of kinase and phosphatase activity, whereas the kinome array measures only kinase activity, the Western blotting data indicate the presence of compensatory mechanisms counteracting increased phosphorylation of substrate proteins. Hence, these data validate the robustness and validity of the kinome data.

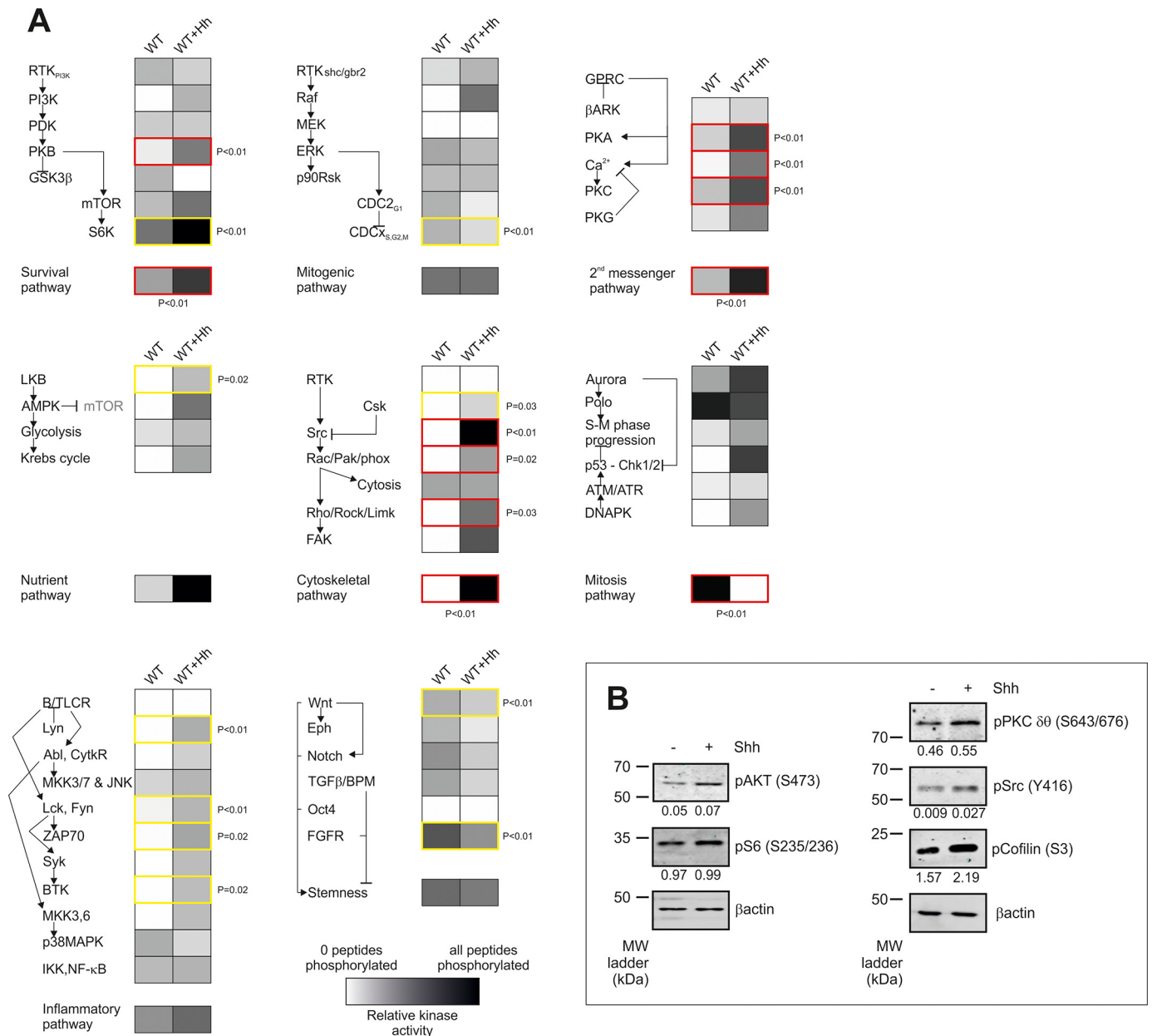
### Patched-dependent Smoothened-independent effects on cellular kinase activity

The existence of Patched-dependent Smoothened-independent signal transduction is supported by various observations (32) and appears highly relevant in that it is essential for cancer stem cell survival in colorectal cancer (31). To test whether such signaling is present in our model system, we incubated embryonic fibroblasts with [ $^3$ H]sucrose (which is membrane-impermeable and is only taken up via endocytosis in most cell types) and challenged the cells with either a vehicle control or 2  $\mu$ g/ml Shh, in the presence or absence of the Smoothened inhibitor cyclopamine (Fig. 3A). We observed strong accumulation of radioactivity in Hedgehog-challenged cells as well as in cells challenged with Hedgehog in the presence of cyclopamine, indicating that Smoothened-independent cellular function is present in Hedgehog-stimulated fibroblasts. As a control, tomatidine (an alkaloid similar to cyclopamine that has no action on Smo) was used, but no effect was observed (not shown). To confirm our observation using a more specific, clinically relevant Shh signaling inhibitor, we used vismodegib. Vismodegib is described as a specific Smoothened inhibitor and was approved by the Food and Drug Administration in 2012 for use in advanced basal-cell carcinoma (33). Vismodegib-treated cells were stimulated with Shh (2  $\mu$ g/ml) and incubated with Lucifer Yellow, a classic fluorescent molecule that can be used to quantify pinocytosis (34). Lucifer Yellow uptake in the presence of Shh was not decreased by inhibition of Smoothened (Fig. 3B). We thus concluded that endocytosis following Hedgehog stimulation does not require Smoothened activity, and that hence our model system was suitable for investigating at least certain aspects of Smoothened-independent signal transduction.

To further characterize these aspects, we performed kinome profiling of *Smoothened*<sup>-/-</sup> fibroblasts (originally obtained from Drs. James Chen and Philip Beachy and previously described by Varajosalo *et al.* (35)), challenged with either a vehicle control or 2  $\mu$ g/ml Shh for 10 min. The results are summarized in Fig. 4A and Table S1 and reveal that the influence of Smoothened-independent Hedgehog-induced signaling on cellular kinase activity is substantial. Lacking, however, is G protein-coupled receptor-associated signal transduction, which is obviously in line with the absence of Smoothened-dependent events. In particular, activation of cytoskeletal remodeling is seen following the addition of Hedgehog, which correlates with a reduced activity of the negative Src activity regulator, Csk. This may relate to the observed Smoothened-independent effects of Hedgehog on endocytosis described above, especially as kinase enzymatic activity directed against focal adhesion kinase-responsive peptides is observed to be co-activated in our profiles, which fits canonical signaling on endocytosis (36). Another prominent effect upon Hedgehog in *Smoothened*<sup>-/-</sup> fibroblasts is increased mTOR activation, whereas inflammatory signal transduction was also activated. Hedgehog in WT fibroblasts provokes similar effects (see above), and thus these effects of Hedgehog signaling appear at least partially to stem from Smoothened-independent signaling. Similarly, activation of Wnt and Notch signaling is also seen, and thus this aspect of Hedgehog signaling seems also independent of Smoothened. Interestingly, in the absence of Smoothened, Hedgehog activates rather than inhibits PKA, and it is tempting to speculate that this effect may relate to activating phosphorylation of Smoothened by PKA that has been described in Hedgehog signaling (37). In conjunction, these results reveal that an unexpectedly large proportion of Hedgehog signal transduction toward the cellular kinome is mediated through noncanonical Patched-dependent Smoothened-independent signaling.

To simulate these Patched-dependent, smoothened-independent effects, we also treated cells with vismodegib in the presence and absence of Shh (Figs. 1 and 4B) and show that Wnt signaling (as measured by  $\beta$ -catenin activity) was also indeed activated independently of smoothened in this system, as were PAK and S6 phosphorylation. Although the changes in phosphorylation observed on Western blotting are more modest than those observed in the kinome array, they do support the peptide array data. As Western blotting measures the sum of kinase and phosphatase activity, whereas the kinome array measures only kinase activity, the Western blotting data indi-

**Figure 1. Outline of the study.** A, technical approach, kinome profiling. In this study, we aimed to comprehensively characterize cellular kinase enzymatic activities. To this end, appropriately stimulated cell cultures were washed with ice-cold PBS and lysed in a nondenaturing complete lysis buffer so as to solubilize cellular kinases. Lysates were then transferred to arrays consisting of a substrate peptide library, the samples were spotted in triplicate to assess technical reproducibility on a hydrogel-coated glass carrier. Upon the addition of radioactive ATP and an activation mix, kinases (if enzymatically active) will phosphorylate substrate peptides. Incorporation of radioactive ATP into a substrate peptide was taken as measure of enzymatic kinase activity toward a particular substrate. The broad variation in specific substrates used (see also the supporting data) allowed us to obtain a more-or-less complete description of cellular signaling, the so-called kinome. B, biological approach. In this study, we first generated a description of the effects of Shh challenge on cellular signaling in general by comparing kinome profiling results of cultures challenged and not challenged by the morphogen. To identify signal transduction events that were downstream of Ptc but did not involve Smo, the Hedgehog-provoked effects on the cellular kinome were studied in fibroblasts genetically deficient for Smo. Finally, to identify events that are solely dependent on the activation of Smo, we studied the effects of the Smo agonist purmorphamine (*purm*). Several kinome profiling results were subsequently validated using a second approach, in which MEFs were stimulated with Shh and subjected to Western blot analysis. To simulate Ptc-dependent effects, cells were treated with the Smoothened inhibitor (vismodegib) prior to Shh stimulation. To simulate Smo-dependent effects, cells were treated with the Smoothened agonist SAG.



**Figure 2. Effects of Hedgehog stimulation on cellular signaling as determined by kinome profiling.** A, murine fibroblasts were stimulated with 2  $\mu\text{g}/\text{ml}$  Shh. Subsequently, cells were lysed, and the resulting lysates were used to phosphorylate arrays of different kinase substrates employing [ $\gamma$ - $^{33}\text{P}$ ]ATP, and radioactivity incorporated in the different substrates was determined. Peptide substrates were allotted to elective signal transduction elements. The figure depicts the number of peptides significantly phosphorylated (which means the number of peptides that received a Markov “on” call; see “Experimental procedures”) for each element. A darker color reflects more kinase activity toward substrate elements, and the results reveal the effects of Hedgehog stimulation on cellular signal transduction; thus, black means that all peptides were significantly phosphorylated, whereas white means that no peptides allotted to this signal transduction in this experimental condition were phosphorylated. Results were first statistically tested by a dichotomous analysis based on the number of Markov “on” calls observed in vehicle- and Shh-stimulated cultures. If statistically significant differences were noted, the signal transduction category is highlighted with a red border, and the level of significance observed is indicated in red. For signal transduction elements in which this very robust analysis failed to detect a statistically significant difference, a parametric test was performed. If this proved significant, the category is highlighted in orange, and the corresponding level of significance is depicted as well. The results provide a wealth of data on the effects of Hedgehog stimulation on cellular signaling. B, MEFs were grown in 6-well plates. To stimulate Smo- and Ptc-dependent signaling, cells were treated with Shh (2  $\mu\text{g}/\text{ml}$ ) for 10 min and compared with unstimulated cells. Cells were lysed, and proteins were resolved by SDS-PAGE followed by blotting to PVDF and incubation of membrane with antibodies against the indicated phosphorylated proteins. Blots were reprobbed with antibodies against  $\beta$ -actin to confirm equal loading.

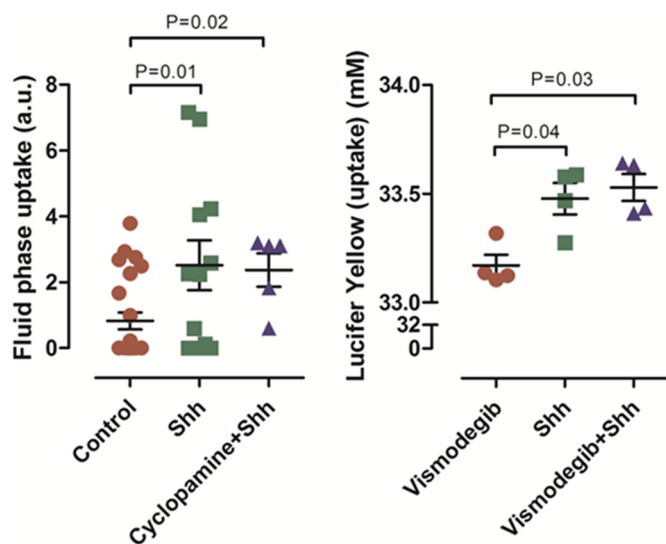
cate the presence of compensatory mechanisms counteracting increased phosphorylation of substrate proteins. In addition, we verified the nature of the Smoothened $^{-/-}$  fibroblasts by Western blotting (Fig. 4C).

These results, demonstrating the presence of a Smoothened-independent activation, suggest that treatment with Smoothened inhibitors may lack the potential to attenuate full Shh signaling and

may provide some explanation as to why, although efficacious in some tumor types, the use of vismodegib in other Shh-activated tumors (e.g. prostate cancer) shows less promise (38).

#### Cellular kinase response to selective Smoothened activation

Next, we decided to investigate the effects of selective Smoothened activation in MEFs. To this end, we challenged



**Figure 3. Effects of Hedgehog on endocytosis and the influence of Smoothened inhibition thereon.** A, fibroblast cultures were grown in 24-well plates and incubated in a 1-ml volume containing 200 nCi of [ $^3$ H]sucrose in the presence or absence of either 1  $\mu$ g/ml Shh and 10  $\mu$ M cyclopamine or appropriate vehicle control. At the end of the experiment, cells were extensively washed with ice-cold PBS and lysed in Nonidet P-40 for subsequent scintillation counting. As sucrose can only enter cells through fluid phase uptake, this provides a reliable measure of cellular endocytosis. We observe that Hedgehog stimulates fluid phase uptake, and this effect does not require Smoothened, as it is not sensitive to the Smoothened inhibitor cyclopamine. B, similarly, fluorescence spectrophotometry indicated that fibroblasts grown in 96-well plates and treated with Shh (2  $\mu$ g/ml) for 6 h still show uptake of Luciferin Yellow (35  $\mu$ M) even in the presence of the smoothened inhibitor vismodegib (50  $\mu$ M), indicative of a Ptc-dependent, Smo-independent cellular process. a.u., arbitrary units; error bars, S.D.

cells with purmorphamine, a purine derivative that acts as a direct agonist of Smoothened (39). The results are provided in Fig. 5A and Table S1. We observe that purmorphamine results in inhibition of PKA. As Hedgehog stimulation in both WT and Smoothened $^{-/-}$  cells was increased, PKA activity appears dominated by Patched-dependent, Smoothened-independent signaling. Intriguingly, purmorphamine results in a down-regulation of ROCK, which is important for a variety of cellular processes, but in particular for cytoskeletal reorganization (40). It was earlier established that Smoothened is a powerful mediator of chemotactic responses, but only so when not located at the primary cilium (30). At the primary cilium, Smoothened loses its capacity to stimulate chemotaxis. The apparent down-regulation of ROCK activity following purmorphamine stimulation is thus best explained by a purmorphamine-dependent recruitment of Smoothened to the primary cilium. The strong canonical responses to purmorphamine stimulation observed by others would agree with this notion, as would the marked down-regulation of PKA activity in our profiles. We also employed the Smoothened agonist SAG to confirm some of these effects by Western blot analysis (Fig. 5B). Whereas generally lower than Shh (Fig. 5B), SAG induced Src, Pak, PKB/S6, and Wnt signaling in MEFs. Although these changes in phosphorylation observed on Western blotting are more modest than those observed in the kinase array, they do support the peptide array data. As Western blotting measures the sum of kinase and phosphatase activity, whereas the kinase array measures only kinase activity, the Western blotting data indicate

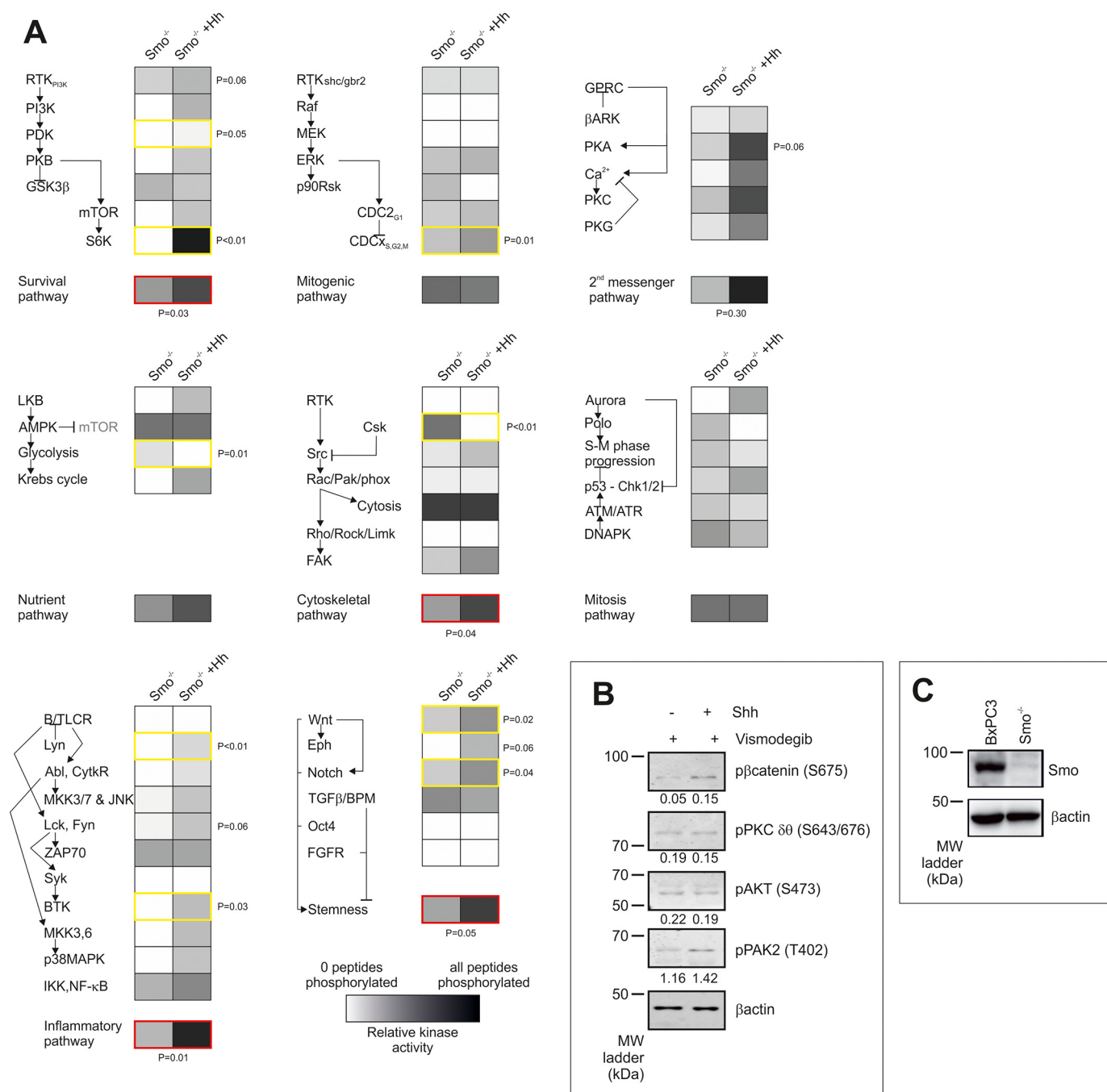
the presence of compensatory mechanisms counteracting increased phosphorylation of substrate proteins.

In some aspects, the rapid Smoothened-independent effects and rapid Smoothened-dependent effects on cellular kinase activities studied in our experimental setup, are similar, as both provoke mTOR activation and, in our model system, activation of Wnt signaling. In this sense, noncanonical signaling downstream of Patched and Smoothened may converge to produce the final phenotype. It is important to stress that our setup does not allow for studying the effects of canonical Hedgehog signaling, which requires transcriptional responses. Generally speaking, canonical signaling and noncanonical signaling by morphogens counteract each other, and the effects observed in this study partially substantiate that notion for Hedgehog signaling as well. Not seen downstream of specific Smoothened stimulation were strong pro-inflammatory responses, which therefore seem mainly Patched-dependent. Generally speaking, Patched-specific signaling events (*i.e.* the effects of Hedgehog stimulation on Smoothened $^{-/-}$  fibroblasts) were more pronounced than those provoked by purmorphamine stimulation, as also evident from the number of peptides that became significantly phosphorylated (see "Experimental procedures") (*i.e.* 180 peptides in Hedgehog-stimulated Smoothened $^{-/-}$  fibroblasts and 134 in purmorphamine-stimulated WT fibroblasts). It thus appears that the major branch of noncanonical Hedgehog signaling is downstream of Patched but not of Smoothened (see Fig. 6 and Table 1 for an overview).

## Discussion

Hedgehog signal transduction is highly unusual, containing many features unique to this signaling system (*e.g.* see Refs. 41 and 42). Apart from canonical Hedgehog signaling, Hedgehog effects in physiology and pathophysiology also depend on so-called noncanonical signaling. For most morphogens, noncanonical signaling has been identified, and the effects observed are, in general, in contrast to the effects derived from canonical signaling. An example is BMP signaling, which generally acts as a tumor suppressor in the colon (5). In the presence of canonical BMP-signaling abrogating SMAD4 mutations, a noncanonical BMP-induced signaling pathway becomes evident that stimulates epithelial-to-mesenchymal transition and metastasis via activation of Rho and ROCK and furthers the colon cancer process (9). Likewise, noncanonical Wnt signal transduction mediates important aspects of the action of this morphogen in the body through activation of small GTPases like Rac, Rho, and Cdc42 to regulate the activity of ROCK, mitogen-activated protein kinase, and c-Jun N-terminal kinase as well as Ca $^{2+}$  signaling, also an effect important for colon cancer metastasis (43). For Hedgehog also, various modes of noncanonical signaling have been described, both downstream of Patched and independent of Smoothened as well as downstream of Smoothened. The most prominent example of the former concerns colorectal cancer stem cells (31). Whereas canonical Gli-dependent Hedgehog signaling negatively regulates Wnt signaling in the normal intestine and intestinal tumors (30), Hedgehog signaling in colon cancer stem cells activates a noncanonical Patched-dependent but Smoothened-





**Figure 4. Effects of Hedgehog stimulation on cellular signaling in *Smo*-deficient fibroblasts.** Murine *Smo*<sup>-/-</sup> fibroblasts were stimulated with 2  $\mu$ g/ml Shh. Subsequently, cells were lysed, the resulting lysates were used to phosphorylate arrays of different kinase substrates employing [ $\gamma$ -<sup>33</sup>P]ATP, and radioactivity incorporated in the different substrates was determined. Peptide substrates were allotted to elective signal transduction elements, and a darker color reflects more kinase activity toward substrate elements. The results reveal the effects of Hedgehog stimulation on cellular signal transduction. Results were first statistically tested by a dichotomous analysis based on the number of Markov "on" calls observed in vehicle- and Shh-stimulated cultures (highlighted with a red border). For signal transduction elements in which this very robust analysis failed to detect a statistically significant difference, a parametric test was performed (highlighted in orange). The results reveal an intricate web of Patched-dependent Smoothened-independent noncanonical signal transduction events. **B**, *Smo*-independent signaling was investigated by treating cells in the presence of both Shh (2  $\mu$ g/ml) and the Smoothened inhibitor vismodegib (50  $\mu$ M, 30 min pre-incubation). Cells were lysed, and proteins were resolved by SDS-PAGE followed by blotting to PVDF and incubation of membrane with antibodies against the indicated phosphorylated proteins. Blots were reprobbed with antibodies against  $\beta$ -actin to confirm equal loading. **C**, validation of the nature of the *Smo*<sup>-/-</sup> culture. BxPC3 cells were used as *Smo*<sup>+/+</sup> control. Cells were lysed, and proteins were resolved by SDS-PAGE followed by blotting to PVDF and incubation of membrane with an antibody against Smo. Blots were reprobbed with antibodies against  $\beta$ -actin to confirm equal loading. *ERK*, extracellular signal-regulated kinase; *MEK*, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

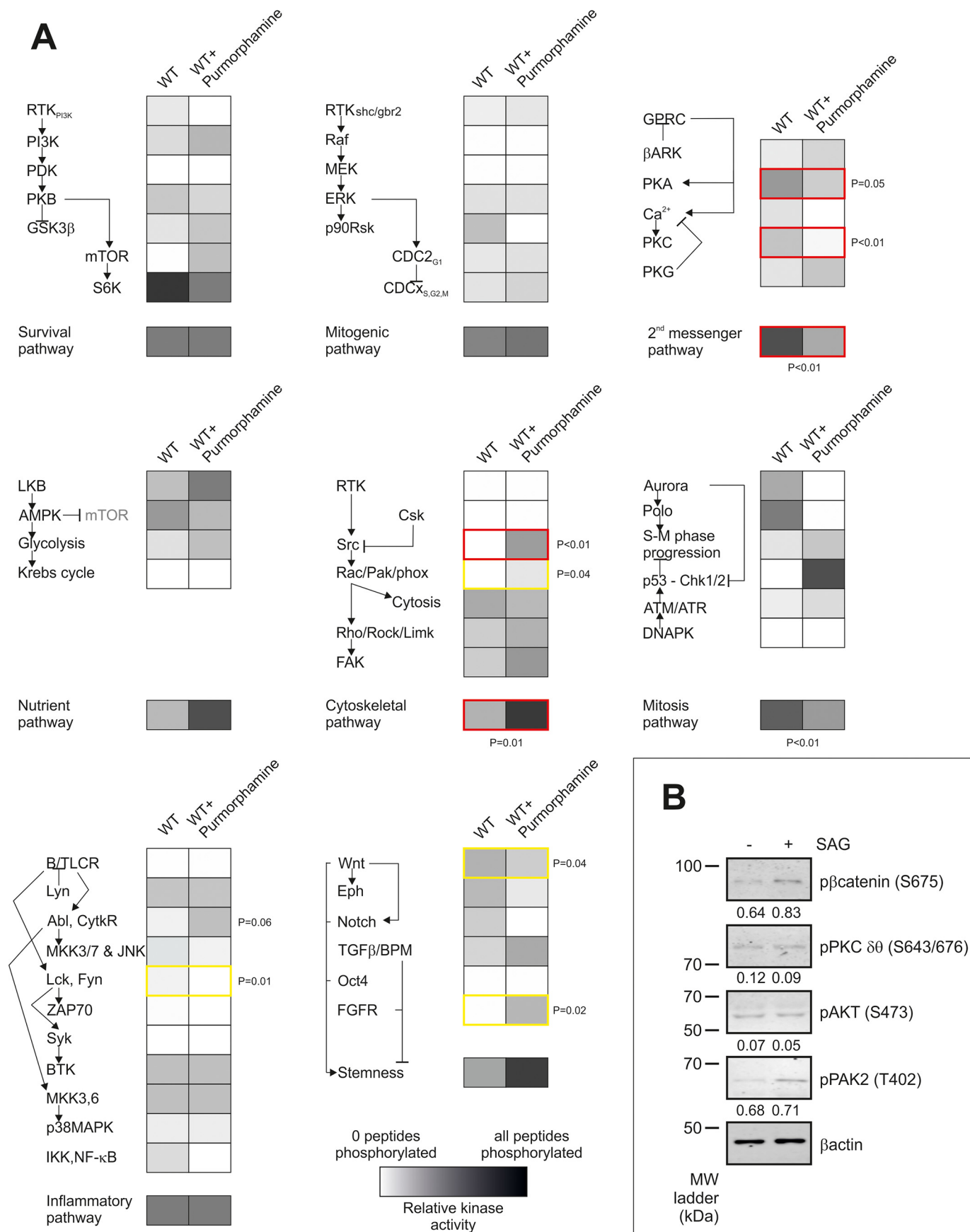
independent signaling that is required for survival of these cancer stem cells.

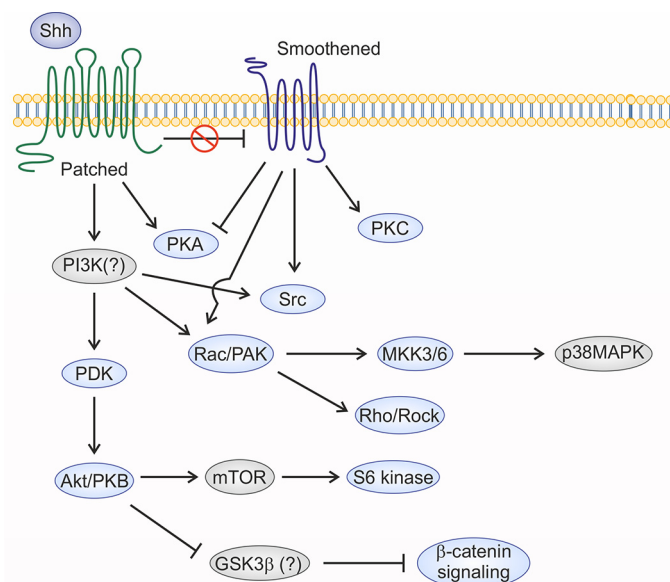
Apart from Patched-dependent Smoothened-independent noncanonical Hedgehog signaling, Smoothened-dependent

Gli-independent noncanonical Hedgehog signaling has also been described, and likewise the molecular mechanisms involved are only partly understood. The interaction of Hedgehog with Patched stimulates the translocation of Smoothened



# Smoothened noncanonical Hedgehog signaling





**Figure 6. Selected kinome profiling-detected Shh-provoked signal transduction events and the role of Patched and Smoothened therein.** Blue elements were confirmed, whereas gray elements showed a trend but did not reach Bonferroni-corrected statistical significance. The results reveal that the role of Patched-dependent Smoothened-independent signal transduction is more prominent in transcription-independent cellular effects of Hedgehog than previously thought.

to the primary cilium, which is required for the transcriptional Hedgehog response (26). This translocation involves activation of phospholipase A<sub>2</sub> following Smoothened activation and results in the enzymatic release of arachidonic acid from plasma membrane phospholipids. Arachidonic acid metabolites are powerful actin cytoskeleton-remodeling agents (44), and although located outside the primary cilium, Smoothened also mediates transcription-independent actin reorganization and chemotactic responses through the production of these metabolites (17–19). The physiological importance of this noncanonical response to Hedgehog signaling is illustrated by its pivotal role via Hedgehog effects in directing neurite projection (18). It has been shown that noncanonical Hedgehog effects on axonal guidance involve activation of Src-like kinases (19), and our data now yield a plethora of information regarding the signaling pathways contributing the noncanonical signaling induced by Hedgehog. The changes in kinase activity measured may derive from either altered expression of kinases or altered activity of the individual kinase enzymes involved. As the stimulation period of the experiments is very short (10 min), we feel the latter explanation is the most probable, but until experiments in the presence of translation inhibitors have been performed, other possibilities should be kept in

mind. Similarly, it should be noted that there is a disconnect between effect size on Western blotting and kinome array, suggesting that part of the kinase effects observed are counteracted by compensatory phosphatase activity; thus, the importance of our observations for phenotypic cellular activities such as proliferation, viability, and migration will remain to be investigated in other studies. Nevertheless, the final effect of Hedgehog in physiology and pathophysiology is resultant from the integration of both canonical and noncanonical Hedgehog signaling (32). The potential of pharmacological inhibitors of Hedgehog signaling in the treatment of disease has received substantial attention, and various trials employing pharmacological inhibitors of Hedgehog signaling have been conducted. Especially vismodegib and sonidegib have met with success in diseases driven by canonical Hedgehog signaling, in particular dermatological cancer (33). Despite the evidence, however, that Hedgehog signaling is important for many gastrointestinal cancers (45), trials in this type of disease have not yet proven successful. In view of our data presented above that Patched and not Smoothened is a major mediator of noncanonical Hedgehog signaling and the momentum-gaining notion that especially noncanonical Hedgehog signaling may be important for maintaining gastrointestinal cancer (31), this may not be surprising. Vismodegib and sonidegib target Hedgehog signaling at the level of Smoothened and leave Patched-dependent noncanonical Hedgehog signaling unaffected. Especially in view of the Patched-dependent Smoothened-independent Wnt signaling, one can easily imagine that especially the noncanonical branch of Hedgehog signaling is important in supporting growth in the gastrointestinal compartment. An implication of our results is thus that future Hedgehog-based therapy with respect to gastrointestinal cancer should be directed at counteracting the interaction of Patched with Hedgehog rather than the current strategy of targeting Smoothened. Obviously, proof of this notion awaits experimentation in cancer cells that are insensitive to Smoothened inhibitors but require extracellular Hedgehog.

## Conclusions

Here we characterize the noncanonical aspect of Hedgehog signaling. We observe that such noncanonical signaling mainly involves Patched-dependent Smoothened-independent signaling, with especially activation of cytoskeletal remodeling and the activation of Wnt signaling being prominent elements. Thus, for efficient targeting of Hedgehog-dependent signaling, it may prove essential to target such signaling at the level of Patched and not Smoothened.

**Figure 5. Effects of selective Smoothened activation by purmorphamine stimulation on cellular signaling in fibroblasts.** A, murine fibroblasts were stimulated with purmorphamine. Subsequently, cells were lysed, the resulting lysates were used to phosphorylate arrays of different kinase substrates employing [ $\gamma$ -<sup>32</sup>P]ATP, and radioactivity incorporated in the different substrates was determined. Peptide substrates were allotted to elective signal transduction elements, and a darker color reflects more kinase activity toward substrate elements. The results reveal the effects of Hedgehog stimulation on cellular signal transduction. Results were first statistically tested by a dichotomous analysis based on the number of Markov “on” calls observed in vehicle- and Shh-stimulated cultures (highlighted with a red border). For signal transduction elements in which this very robust analysis failed to detect a statistically significant difference, a parametric test was performed (highlighted in orange). The results reveal a web of Smoothened-dependent signal transduction events clearly distinct from Patched-dependent signaling. B, to investigate Ptc-independent signaling, cells were subjected to treatment with the Smo agonist SAG (100 nM) for 10 min. Cells were lysed, and proteins were resolved by SDS-PAGE followed by blotting to PVDF and incubation of membrane with antibodies against the indicated phosphorylated proteins. Blots were reprobed with antibodies against  $\beta$ -actin to confirm equal loading. ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase.

**Table 1**

**Summary of pathways analyzed using kinome**

Shown is a cross-comparison as a short description of kinome profiling, showing the major pathways and a statistical comparison of the conditions for canonical and noncanonical pathways (Patched (Ptc)-dependent and Smoothened (Smo)-dependent).

| Pathway          | Shh canonical | Statistics | Ptc-dependent | Statistics | Smo-dependent | Statistics |
|------------------|---------------|------------|---------------|------------|---------------|------------|
| Survival         | +             | <0.01      | +             | 0.03       |               |            |
| Mitogenic        |               |            |               |            |               |            |
| Second messenger | +             | <0.01      |               |            | +             | 0.01       |
| Nutrient         |               |            |               |            |               |            |
| Cytoskeletal     | +             | <0.01      | +             | 0.04       |               |            |
| Mitosis          | –             | 0.02       |               |            |               |            |
| Inflammatory     |               |            | +             | 0.01       |               |            |
| Stemness         |               |            | +             | 0.05       |               |            |

## Experimental procedures

### Materials

Cyclopamine was from Biomol (Hamburg, Germany). Purmorphamine was from EMD Biochemicals (Darmstadt, Germany) and was dissolved in ethanol (final concentration 0.2%). Recombinant Sonic HedgehogN was from R&D Systems. Sonic Hedgehog inhibitor vismodegib (GDC-0449) was from Selleck Chemicals and reconstituted in DMSO (final concentration 0.025%). Shh agonist SAG (SML1314-1MG; catalog no. 14454) was from Sigma-Aldrich, and recombinant murine Shh (315-22, 0513521) was from PeproTech, Inc.

### Cell culture

Smoothened<sup>−/−</sup> fibroblasts (provided by Dr. J. Taipale) and WT mouse embryonic fibroblast (provided by Dr. M. P. Scott) were cultured in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen) and propagated at 37 °C in a 5% CO<sub>2</sub> humidified atmosphere. For experiments, a confluence of 50% cells was allowed to grow in 6-well plates. Stimulations were done, if appropriate, with 2 μg/ml Shh for 10 min. Each experiment consisted of three biological replicas of experiments containing three technical replicas.

### Kinome profiling

For peptide array analysis, we employed the Pepchip kinomics array. The protocol and associated analysis has been described in detail elsewhere (25) and is based on the original protocol of van Baal *et al.* (46). In short, cells were washed in ice-cold PBS and lysed in a nondenaturing complete lysis buffer (cells were lysed in 50 μl of lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM MgCl<sub>2</sub>, 1 mM glycerophosphate, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM NaF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM PMSF). Subsequently, the cell lysates were cleared by centrifugation, and peptide array incubation mix was produced by adding 10 μl of activation mix (50% glycerol, 50 μM ATP, 0.05% (v/v) Brij-35, 0.25 mg/ml BSA) and 2 μl of [ $\gamma$ -<sup>33</sup>P] ATP (~1000 kBq (Amersham Biosciences, AH9968). Next, the peptide array mix was added onto the chip, and the chip was kept at 37 °C in a humidified stove for 90 min. Subsequently, the peptide array was washed twice with TBS with Tween 20, twice in 2 M NaCl, and twice in demineralized H<sub>2</sub>O and then air-dried. The chips were exposed to a phosphor screen for 72 h, and the density of the spots was measured and analyzed with array software (ScanAnalyze). Using grid tools, spot density and

individual background were corrected, and spot intensities and background intensities were analyzed. Data from at least nine independent data points were exported to an Excel sheet for further analysis. Control spots on the array were analyzed for validation of spot intensities between the different samples. Inconsistent data (*i.e.* S.D. between the different data points >1.96 of the mean value) were excluded from further analysis. For each peptide, the average and S.D. of phosphorylation were determined and plotted in an amplitude-based hierarchical fashion. For data analysis, first every peptide was given an “on” call or “off” call (Markov state analysis). To this end, first an average signal was calculated for each peptide using the three biological replicates (each consisting of two technical replicates), yielding an aggregate data set for each of the hematopoietic subsets. Subsequently, for each of the aggregate datasets, either “on” calls or “off” calls were given to each peptide substrate (Markov state analysis). To do this, we assumed that the subset of signals representing the  $1 - e^{-1}$  fraction of peptides having the lowest phosphorylation of all peptides contained pure noise and did not represent meaningful phosphorylation. The distribution of this noise was fitted as a single exponent, using the amplitude-sorted row number of these substrates as the X domain of the distribution, and this single exponent was assumed to describe noise for the entire data set. Now for all data points within the subset, when the actual amplitude observed was  $-1.96$  and the S.D. was in excess of the value expected from distribution describing the noise, a substrate was given an “on” call ( $p < 0.05$ ) in this Markov analysis. Subsequently, results were collapsed on elective signal transduction categories and subjected to dichotomous significance analysis, contrasting Shh-stimulated cultures to parallel vehicle cultures or purmorphamine-stimulated cultures to parallel unstimulated cultures. If a significant result ( $p < 0.05$ ) was detected, we considered the result as robust evidence of differential activation of signal transduction between Hedgehog-stimulated and unstimulated cultures, and in the depiction of results, the corresponding signal transduction categories have been highlighted with a *red border*. For those signal transduction categories in which using this dichotomous testing based on number of Markov state “on” peptides did not result in statistical significance, the relative levels of phosphorylation were also tested using a paired *t* test, directly parametrically comparing phosphorylation of the corresponding spots. As we considered thus-discovered statistically significant differences between the relevant experimental conditions less robust, in the depiction of the results, they have been highlighted with an *orange border*. Note that due to differences in



the number of peptides allotted to the signal transduction categories, apparently large differences in phosphorylation do not always yield statistically significant results, whereas smaller differences can produce such results if the number of substrates in such categories is large.

### Endocytosis assay

Cells were grown on 24-well plates to 70% confluence and were stimulated with either 1  $\mu$ g/ml Shh or vehicle control (0.1% BSA/PBS) and or cyclopamine (Biomol, Plymouth Meeting, PA) for 1 h. After extensive washing with ice-cold PBS, cells were lysed in 1% Nonidet P-40, the lysate was transferred to 4 ml of scintillation fluid, and activity was determined on a Packard Tri-Carb scintillation counter (PerkinElmer Life Sciences). Values were corrected for solvent control-treated cells on ice.

### Lucifer Yellow assay

Mouse embryonic fibroblast were plated at a density of  $3.5 \times 10^3$  cells/well. After 24 h, vismodegib was added (50  $\mu$ M DMSO, 0.25%) for 15 min, followed by Shh treatment at 2  $\mu$ g/ml for 15 min. A stock solution of Lucifer Yellow CH dilithium salt (Sigma-Aldrich, St. Louis, MO) was prepared in PBS, and working solution was prepared in culture medium. The assay was performed using 35 mM Lucifer Yellow, incubated for 6 h at 37 °C and 5% CO<sub>2</sub>. After that, the supernatant was removed, and the Lucifer Yellow fluorescence was measured by spectrophotometer CytoFluor MultiWell Plate 4000 (PerSeptive Biosystems) with excitation at 430 nm and emission at 530 nm. The concentration was calculated using a Lucifer Yellow curve.

### MEF treatment

MEFs were seeded at  $1 \times 10^3$  cells/well, and the next day, cells were incubated with vismodegib (50  $\mu$ M DMSO 0.25%) for 1 h. After, Shh at 4  $\mu$ g/ml and SAG at 100 nM were added for 7 min, and Western blotting samples were prepared, as described below.

### Western blotting

After treatment, the samples were prepared by adding 2 $\times$  Laemmli buffer (100 mM Tris-HCl (pH 6.8), 200 mM DTT, 4% SDS, 0.1% bromophenol blue, and 20% glycerol), and samples were boiled for 95 °C for 10 min. Cell extracts were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Merck Chemicals BV, Amsterdam, The Netherlands). Membranes were blocked in 50% Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) in TBS and incubated overnight at 4 °C with primary antibody. Primary antibodies were as follows: from Cell Signaling, phospho-Akt (Ser-473) (catalog no. 4060S), phospho-PKA C (Thr-197) (catalog no. 4781), phospho-Src family (Tyr-416) (catalog no. 2101), phospho-PKC $\delta/\theta$  (Thr-638/641) (catalog no. 9376), phospho-S6K ribosomal (Ser-235/236) (catalog no. 4858), phospho- $\beta$ -catenin (Ser-675) (catalog no. 9567), and phospho-PAK2 (Ser-20) (catalog no. 2607); from Santa Cruz Biotechnology, Inc.,  $\beta$ -actin (C4) (sc-47778); and from SignalWay, phospho-cofilin (Ser-3) (catalog no. 11139) and phospho-ROCK2 (Ser-1379) (catalog no. 13005). Goat polyclonal anti-Smo C-17 was obtained from Santa Cruz Biotechnology. After washing in TBS-T, mem-

branes were incubated with IRDye® antibodies (LI-COR Biosciences) for 1 h. Detection was performed using an Odyssey reader, and analysis was done using the manufacturer's software.

### Statistical analysis

Statistical analysis details for each experiment are described in the figure legends. Furthermore, statistical methods were: (a) unpaired and paired Student's *t* test, confidence interval at 95%, two-tailed, and (b) one-way analysis of variance repeated-measures test, significance level  $\alpha = 0.05$  (95% confidence interval), followed by Tukey's post-test.

**Author contributions**—The head group leaders M. P. P., M. F. B., M. J. B., and C. A. S. contributed to the article conceptualization; the principal group researchers K. P. and G. M. F. were responsible for designing the methodology; A. V. S. F., A. I. A., W. C., and L. B. performed the research investigation; A. I. A. was responsible for the original writing; and A. V. S. F., M. P. P., G. M. F., and M. F. B. were responsible for reviewing and editing. The work was supervised by M. P. P. and C. A. S. All authors read and approved the final manuscript.

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## Smoothed-dependent and -independent pathways in mammalian noncanonical Hedgehog signaling

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